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NO. 462

INHIBITION OF THE ETHANOL-INDUCED PRESYNAPTIC RELEASE OF ACETYLCHOLINE BY HEMICHOLINIUM-3 (U)

by

John G. Clement, PhD.

PROJECT NO. 13D03

June 1977

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ABSTRACT

Various concentrations of ethanol (0.29 - 1.09 M) potentiated the indirect supramaximal twitch in the rat diaphragm (RD) and chick biventer cervicis (CBC) preparations. Ethanol (1.09 M) produced a contraction of the muscle. Ethanol (0.43 M - 0.72 M) was found to antagonize the curare neuromuscular (NM) blockade but not the hemicholinium -3 (HC-3) blockade in both preparations. However, if ethanol was applied after washout of HC-3 a potentiation of the twitch response was noted. Pretreatment of the RD for various times with various concentrations of ethanol was found to protect against curare but not HC-3 NM blockade. Tetraethylammonium produced results similar to ethanol in that it antagonized the curare blockade but not the HC-3 NM blockade on the CBC and RD preparations. The results suggest that HC-3 but not curare inhibits the ethanol-induced release of acetylcholine from the presynaptic nerve terminal.

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INTRODUCTION:

Ethanol increased the amount of acetylcholine (ACh) released by nerve impulses as well as enhanced the depolarizing action of the neurotransmitter on the post-synaptic membrane in the rat diaphragm (Gage, 1965) and frog skeletal muscle (Inoue and Frank, 1967). A decrease in muscle contractility was also observed. Ethanol also potentiated the indirect muscle twitch response of frog sciatic nerve-gastrocnemius preparation (Sachdev et al., 1963) and the action of ACh and carbachol on eserinized and non-eserinized frog rectus abdominis muscles (Nelemans, 1962; Sachdev et al., 1963). These potentiating actions of ethanol on muscle contractions were considered independent of any anticholinesterase activity (Sachdev et al., 1963; Gage, 1965).

Ethanol has been reported to have a decurarizing effect (Feng and Li, 1941; Boobis et al., 1975) and to potentiate the end-plate potential recorded in the presence of curare (Gage, 1965; Inoue and Frank, 1967; Okada and Adachi, 1962). Boobis et al. (1975) found that pretreatment of mice with ethanol would protect them against the toxic effects of hemicholinium-3 (HC-3) and d-tubocurarine (curare). From in vitro studies they suggested that the anti-curare activity of ethanol was due to either a depolarizing effect on the post-synaptic membrane or

an increase in the release of neurotransmitter. This study was undertaken to investigate further the nature of the antagonistic action of ethanol on curare and HC-3 neuromuscular (NM) blockade, reported by Boobis et al. (1975).

METHODS AND MATERIALS:

Female rats were decapitated and exsanguinated. The phrenic nerve-rat diaphragm (RD) prepared according to Bülbring (1946) was suspended in a 60 ml organ-bath containing Krebs-Henseleit solution of the following composition (mM): NaCl, 118; KCl, 4.72; $CaCl_2$, 2.52; $MgSO_4$, 1.18; KH_2PO_4, 1.18; $NaHCO_3$, 25.0; and dextrose, 11.1; it was maintained at 37°C and gassed with oxygen containing 5% CO_2. The phrenic nerve was stimulated indirectly with supramaximal square wave pulses of 0.1 msec duration. Contractions were recorded using an isometric force transducer and an ink writing dynograph. The diaphragm was stimulated indirectly for 30 min prior to commencing an experiment. The NM blocking action of curare-type compounds increased upon repeated exposure to the same dose, thus in this study the preparation was exposed to at least four doses of either curare or HC-3 until consistent responses were obtained before actual data gathering experiments were initiated.

In some experiments high-Mg^{++} Krebs was used to block normal NM transmission. The composition of the high-Mg^{++} Krebs solution was as follows (mM): NaCl, 100: KCl, 4.72: $CaCl_2$, 2.52; $MgSO_4$, 17.7; KH_2PO_4, 1.18; $NaHCO_3$, 25.0; and dextrose, 11.1. The diaphragms were initially suspended in normal Krebs and stimulated for 30 min to equilibrate the preparation and determine that it was functioning normally. After this time the normal Krebs was replaced by high-Mg^{++} Krebs. Upon completion of the experiment the high-Mg^{++} Krebs was replaced with normal Krebs and the twitch height allowed to return to normal. If it did not the results were discarded.

Chick Biventer Cervicis Preparation:

The chick biventer cervicis (CBC) muscle as described by Ginsborg and Warriner (1960) was used. The muscle was suspended in a

bath containing Krebs-Henseleit (previously described) solution which was maintained at 37°C and gassed with oxygen containing 5% CO_2. Supramaximal twitches were produced by stimulating the nerve within the muscle tendon with square wave pulses of 0.1 msec duration. Contractions were recorded using an isometric force transducer connected to an ink-writing dynograph. The muscles were stimulated at a rate of 18/min.

Materials:

Double distilled ethanol (95%) was added directly to the organ bath to give the required final concentration. The following chemicals were obtained: d-tubocurarine (ICN); HC-3 (Aldrich); tetraethylammonium (TEA; BDH); carbachol and hexamethonium (Sigma). All drug concentrations refer to final concentrations in the organ-bath. The results were analysed by the Student "t" test and $p < 0.05$ was considered significant.

Results:

The results in Fig. 1 illustrate the effect of various concentrations of ethanol on the supramaximal twitch response of the RD and CBC preparations. The CBC preparation was more sensitive to twitch potentiating and contractile properties of ethanol than the RD preparation. Ethanol at concentrations of 0.29 M, 0.43 M, 0.54 M and 1.09 M potentiated the twitch response in the CBC preparation 4%, 38%, 85% and 92%, respectively, compared to 8%, 27%, 19% and 15% in the RD preparation, respectively. In both preparations a contraction of the muscle was noted following administration of ethanol (1.09 M) which amounted to 50% of the twitch height in the CBC preparation and 27% in the RD preparation following a 1.0 min exposure.

The twitch potentiation induced by ethanol (1.09 M) was transient in both preparations. The twitch response of the RD preparation was completely inhibited following an 1.0 min exposure to 1.09 M ethanol whereas the CBC preparation was not so affected. Following washout of ethanol the twitch response of the RD quickly returned to normal. (Fig. 1).

The results in Fig. 2 illustrate the effect of ethanol on the HC-3 or curare NM blockade in the CBC and RD preparations. In both

preparations ethanol was capable of causing a very prominent potentiation of the twitch response in curare-treated muscles (approximately 77% and 140% twitch potentiation in the RD and CBC preparations, respectively) but this effect was not evident in the HC-3 treated muscles. On the CBC preparation treated with HC-3 ethanol produced a slight potentiation (11%) of the twitch response.

In the next set of experiments the significance of AChE on the anti-curare action of ethanol in the CBC preparation was evaluated. It was found that, when AChE was inhibited by pyridostigmine (1×10^{-6} M), ethanol was still capable of potentiating the twitch following treatment with curare (Fig. 3), thus demonstrating that the ethanol potentiation was not due to AChE inhibition.

In curare-treated preparations it appeared that the ethanol potentiation of the twitch response was greater than that obtained in the control situation (see Fig. 1) whereas HC-3 was inhibiting the twitch potentiating actions of ethanol. Administration of a dose of ethanol (0.72 M) which had no potentiating properties in the presence of HC-3 (3.3×10^{-4} M) (Fig. 4, Top) was capable of restoring the twitch response following the washout of HC-3 (Fig. 4, Middle). This effect was transient because if the ethanol was not washed out after 1.0 min the twitch potentiation gradually faded and the muscle took a longer time to restore its normal twitch response compared to one which did not receive this particular treatment (Fig. 4, Bottom).

In the next series of experiments various concentrations of ethanol were added to RD preparations at various times before the addition of either HC-3 or curare. The results in Table I demonstrate that pretreatment with ethanol offered some protection against the NM blocking properties of curare (3×10^{-6} M) but not HC-3 (3.3×10^{-4} M) as evidenced by the increased time to obtain a 50% NM block in preparations so treated.

The previous results suggested that ethanol was antagonizing the NM blockade by enhancing the release of ACh. To test these possibilities normal NM transmission was completely blocked in some RD preparations by elevating the Mg^{++} concentration to 17.7 mM. The results in Fig. 5

demonstrate that various concentrations of ethanol restored NM transmission in high Mg^{++}-blocked RD preparations. Ethanol (0.29 M) was the most effective concentration in restoring NM transmission. The lower concentration (0.145 M) did not produce as great a response and the higher concentration (0.435 M) produced only a transient restoration of NM transmission. Neither neostigmine methylsulfate nor pyridostigmine bromide (1×10^{-5} M) restored NM transmission, indicating that ACh release was completely blocked in response to nerve stimulation in high Mg^{++}-blocked preparations and also that this effect of ethanol was not due to an anti-AChE action. Normal NM transmission was not restored in Mg^{++}- blocked preparations probably due to the fact that high Mg^{++} concentrations have a depressant effect on the post synaptic responses to ACh (Del Castillo and Engeback, 1954).

TEA was known to produce an increase in transmitter release (Collier and Exley, 1963) therefore its effects were compared to ethanol for antagonism of the HC-3 and curare NM blockade in the CBC and RD preparations. Fig. 6 illustrates the potentiation of twitch response produced by various concentrations of TEA in the RD and CBC preparations. The results in Fig. 7 illustrate that TEA (7.95×10^{-4} M) antagonized the curare NM blockade but had absolutely no effect on the HC-3 NM blockade in both the RD and CBC preparations.

HC-15 was investigated to see if it acted similarly to HC-3. HC-15 (1.16×10^{-3} M) did not have any NM blocking activity in the CBC preparation and did not prevent the ethanol-induced potentiation of the indirect twitch response.

DISCUSSION:

Similar to the results of previous investigators (Sachdev et al., 1963; Etessami, 1972; Boobis et al., 1975; Cooper and Dretchen, 1975) it was found that ethanol potentiated supramaximal indirect muscle contractions while higher concentrations produced a rapid NM blockade. The NM inhibition produced at a high concentration of ethanol was probably the result of a direct effect on muscle contraction. Inoue and

Frank (1967) suggested that it was due to inhibition of the increase in sodium conductance which follows stimulation of the muscle cell.

The results of the ethanol protection experiments and the similarity in action to TEA combined with the fact that ethanol restored NM transmission in high-Mg^{++}-blocked RD preparations suggested that ethanol was promoting an increase in the release of ACh from presynaptic nerve terminals confirming the results of previous investigators (Gage, 1965; Inoue and Frank, 1967).

The results of this study also demonstrated that HC-3 has presynaptic activity other than blocking choline uptake, ie., it appears to block the increase in ACh release produced by ethanol and TEA. This was evident when comparing the effects of ethanol on curare and HC-3 treated CBC and RD preparations to the effect of TEA, a known promotor of ACh release (Collier and Exley, 1963). A direct inhibitory effect of HC-3 on ACh release was previously reported by Hata et al. (1975). Takagi et al. (1970) reported that HC-3 exerted a depressive action on the nicotinic receptor sites on presynaptic nerve terminals. This inhibitory effect of HC-3 on ACh release would explain why Marshall (1969) found that the choline reversal of the curare block was more pronounced than that of the HC-3 blockade. Choline was probably acting in a similar manner to ethanol and TEA in promoting an increased release of ACh possibly by interaction with the presynaptic ACh receptor. However, in the presence of HC-3 this effect would not be as great due to HC-3's effect on blocking the increase in release of ACh.

Previous investigators (Gage, 1965; Inoue and Frank, 1967) demonstrated that ethanol increased the release of ACh at peripheral sites. Inhibition of Na^{+}-K^{+}-Mg^{++}-activated ATP'ase has been reported to enhance ACh release (Paton et al., 1971; Vizi, 1972; Vizi, 1973) and ethanol has been reported to inhibit Na+-K+-Mg++-activated ATP'ase in rats and guinea pigs (Israel et al, 1965; Sun and Samorajski, 1970). Hurwitz et al. (1962) suggested that ethanol was acting as a calcium antagonist and Gothert and Thielecke (1976) suggested that at high concentrations ethanol inhibited calcium influx into sympathetic nerve terminals. This assumption may still be appropriate since Ca^{++} ions are

not essential for ACh release (Quastel et al., 1974) in situations where inhibitors of membrane ATP'ase were used to enhance ACh release (Vizi, 1972). Boobis et al. (1975) found that pretreatment with ethanol protected mice against the toxic effects of HC-3 and tubocurarine. The present hypothesis is that in vitro ethanol is increasing the release of ACh at peripheral sites possibly through inhibition of Na^{+}-K^{+}-Mg^{++}-activated ATP'ase thus protecting the post-synaptic receptors from inhibition by d-tubocurarine. Ethanol has very little depolarizing action at concentrations which block action potential production (Inoue and Frank, 1967) thus this cannot account for the protective action of ethanol. However, the mechanism of the protective effect of ethanol against HC-3 toxicity in mice is different from that against curare suggesting that the mechanisms of NM blockade by HC-3 and curare are different and not similar as reported by Boobis et al. (1975). The protective action of ethanol against HC-3 toxicity remains to be determined.

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TABLE I

EFFECT OF PRETREATMENT TIME AND CONCENTRATION OF ETHANOL ON THE TIME (MIN) TO PRODUCE 50% NM BLOCKADE IN THE RD PREPARATION EXPOSED TO CURARE (3×10^{-6} M) OR HC-3 (3.3×10^{-4} M)

Conc of Ethanol (M)	Time (min) of Pretreatment	NM Blocker	Time (min) to 50% Block[1]	N
-	-	curare	1.4 ± 0.4	7
0.29	1.0	curare	3.4 ± 0.6*	6
0.145	1.0	curare	2.5 ± 0.7*	6
0.29	7.0	curare	3.1 ± 0.9**	5
-	-	HC-3	1.3 ± 0.2	4
0.29	1.0	HC-3	1.0 ± 0.1 n.s.	4

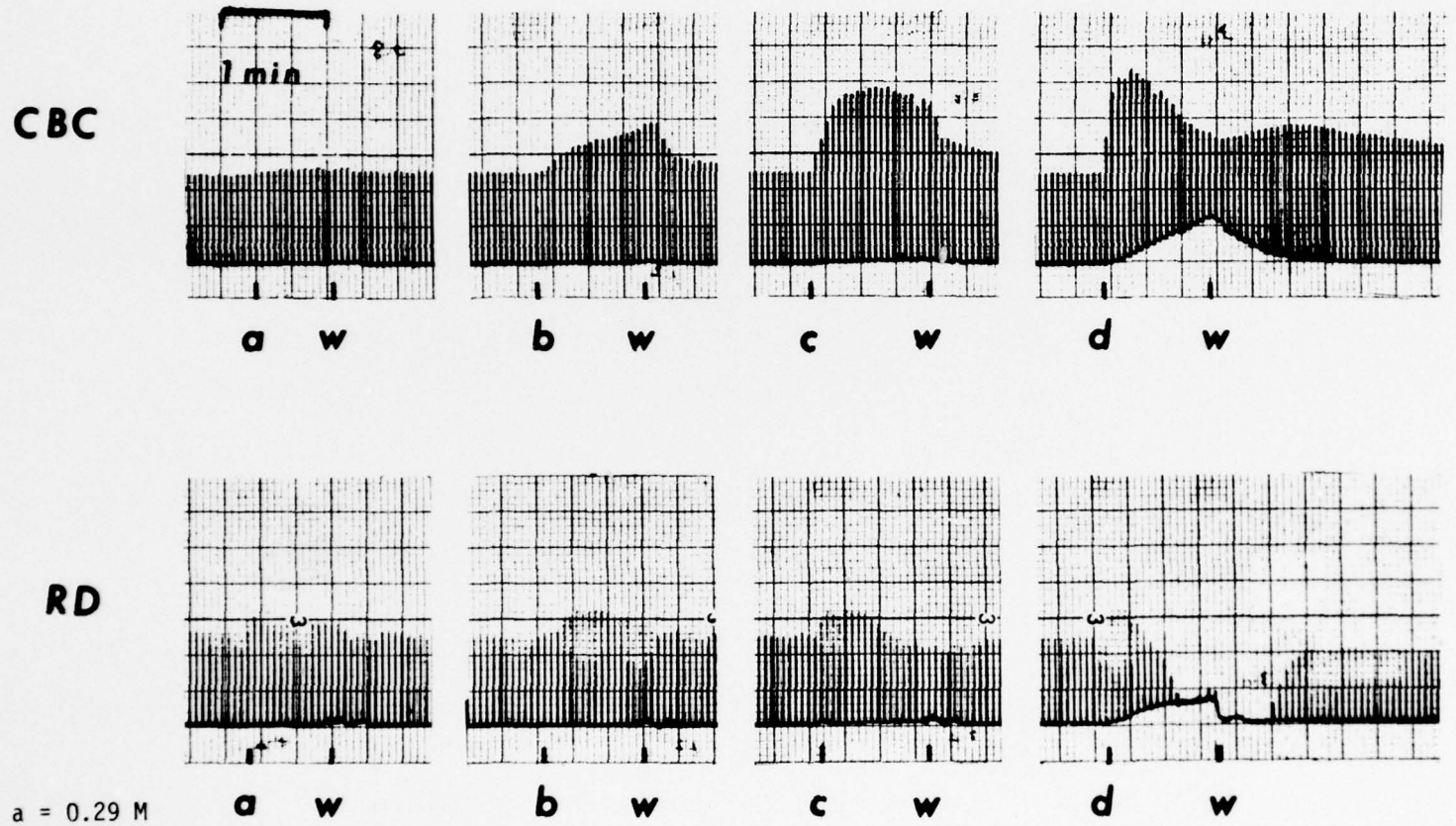
[1] mean ± SEM

significance of difference using Student "t" test.

* $p < 0.001$

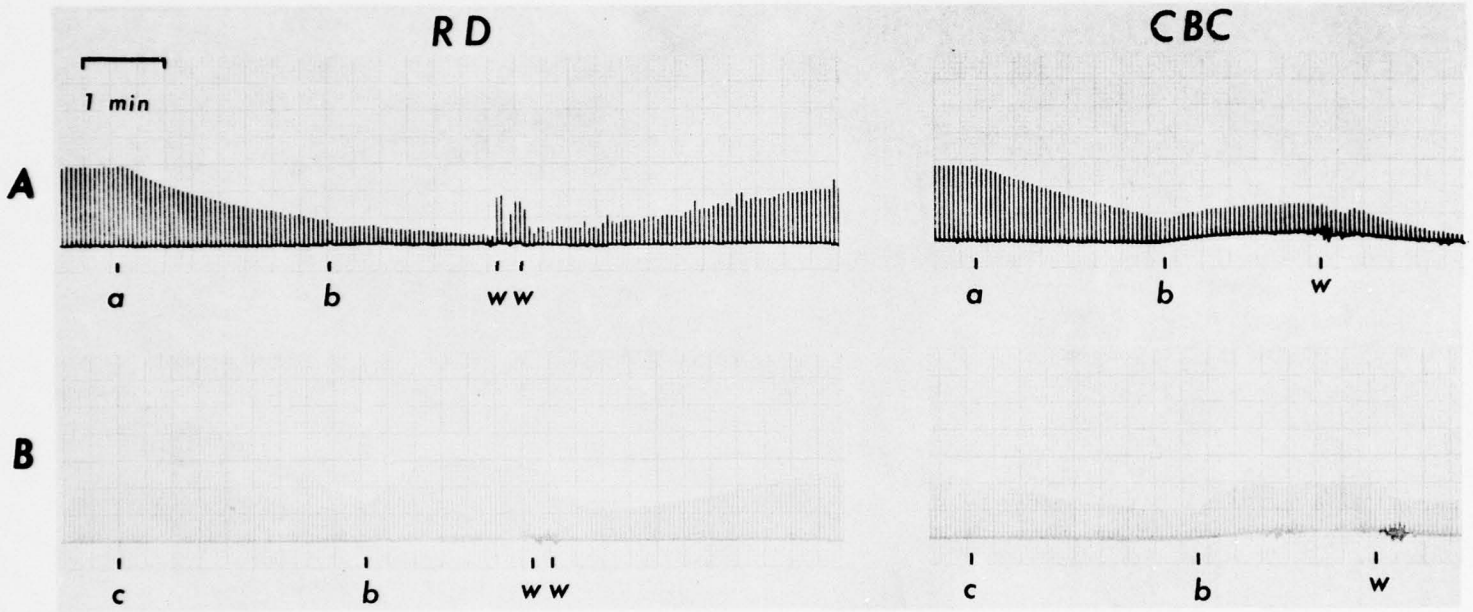
** $p < 0.01$

n.s. = not significant



a = 0.29 M
b = 0.43 M
c = 0.72 M
d = 1.09 M
W = wash

Figure 1: Effect of Various Concentrations of Ethanol on the Supramaximal Twitch in the CBC and RD Preparations.



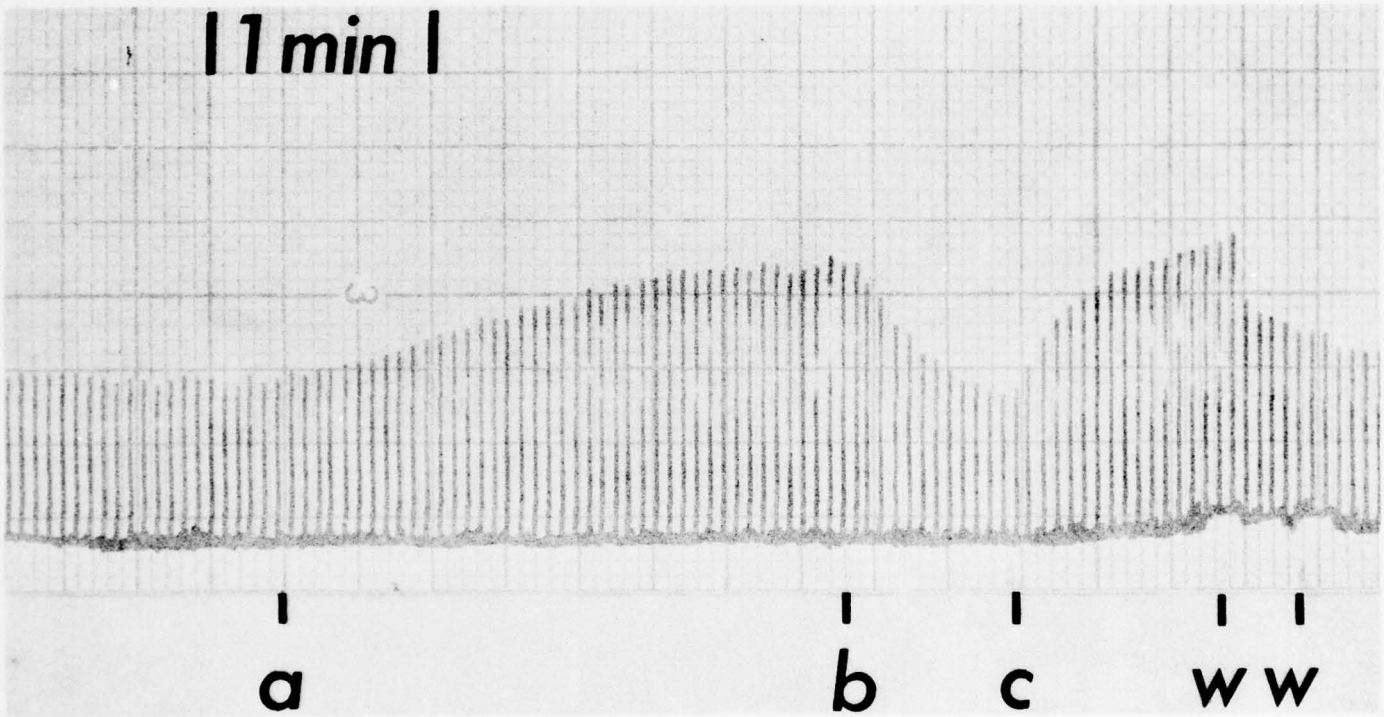
A: HC-3 NM Blockade:

a = HC-3 (3.3 and 1.67 x 10^{-4} M on RD and CBC, resp.)
b = ethanol (0.434 M and 0.72 M on RD and CBC, resp.)
W = wash

B: Curare NM Blockade:

c = d-tubocurarine (3.6 x 10^{-6} M and 6.4 x 10^{-5} M on RD and CBC, resp.)
b = ethanol (same as in A)

Figure 2: Effect of Ethanol on the HC-3 and Curare NM Blockade in the CBC and RD Preparations.



a. Pyridostigmine, 1 x 10^{-6}M

b. d-tubocurarine, 1.58 x 10^{-6}M

c. ethanol, 0.36 M

Figure 3: Effect of Inhibition of AChE on the Anti-Curare Activity of Ethanol in the CBC Preparation.

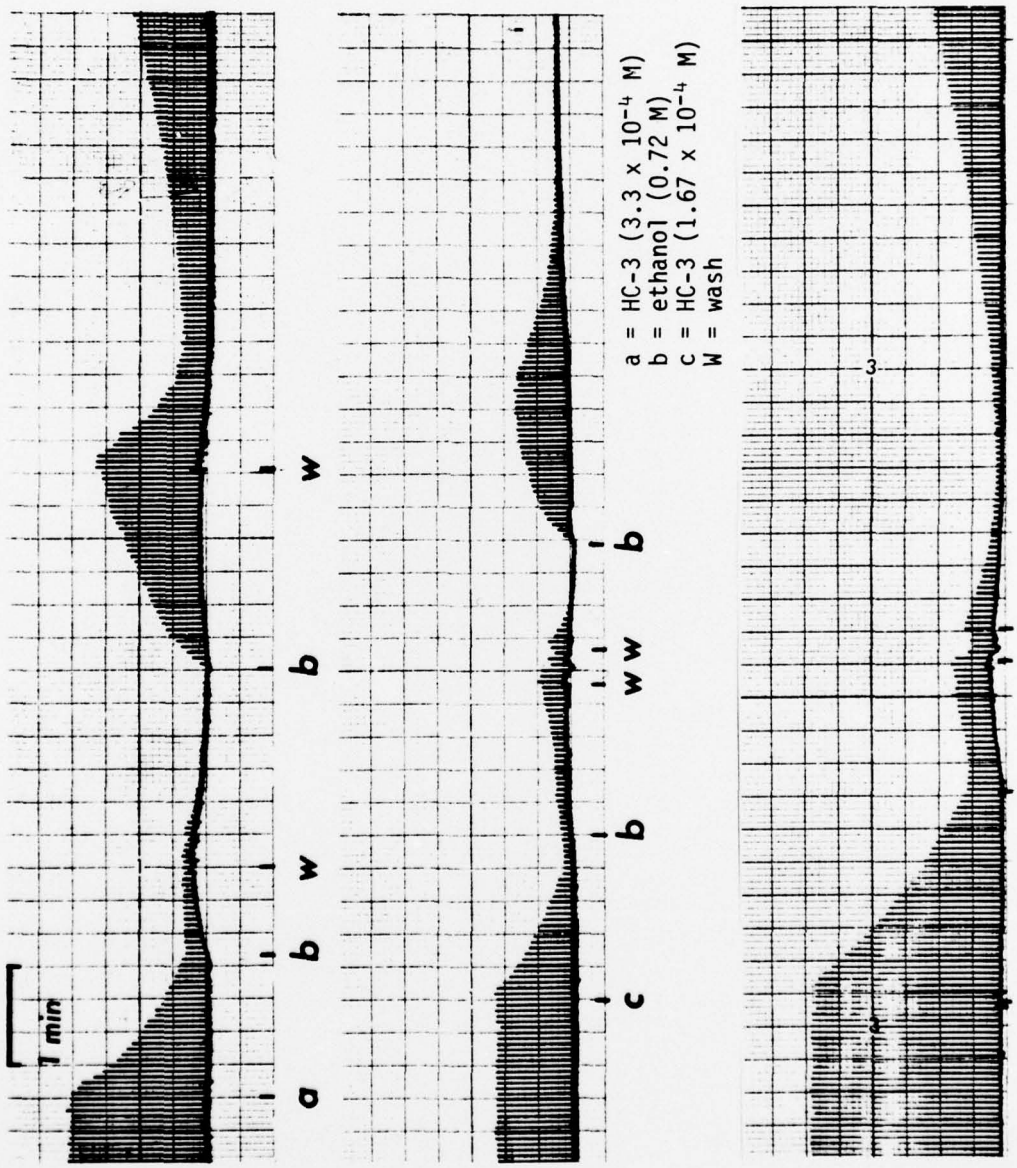


Figure 4: Effect of Ethanol on the Twitch Response of the CBC Preparation in the Presence of HC-3 and Following Washout.

Mg = Twitch response completely inhibited in High Mg^{++} Krebs, $[Mg^{++}]$ = 17.7mM.

a = Ethanol, 0.145 M

b = Ethanol, 0.29 M

c = Ethanol, 0.58 M

W = wash

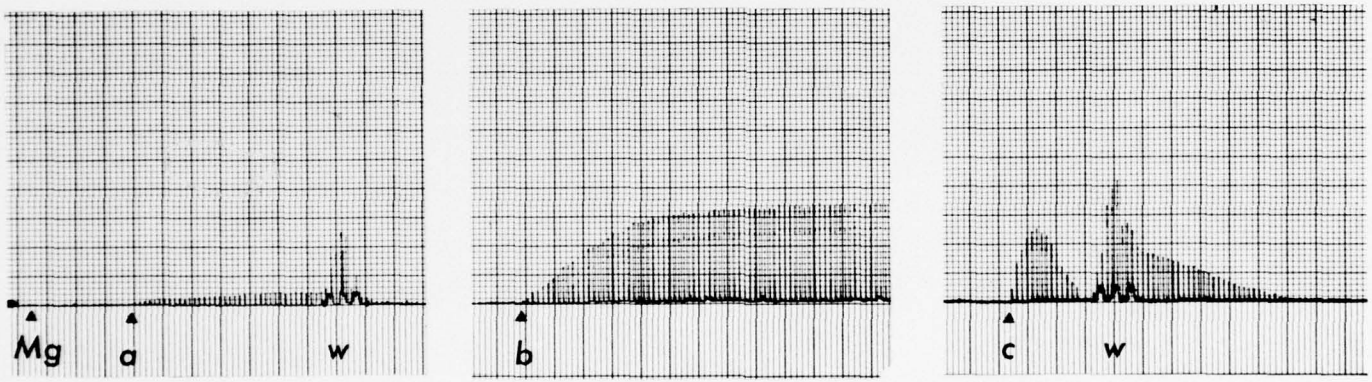


Figure 5: Inhibition of the Twitch Response in the RD Preparation by High Mg^{++} Krebs: Restoration of the Twitch Response Following Ethanol.

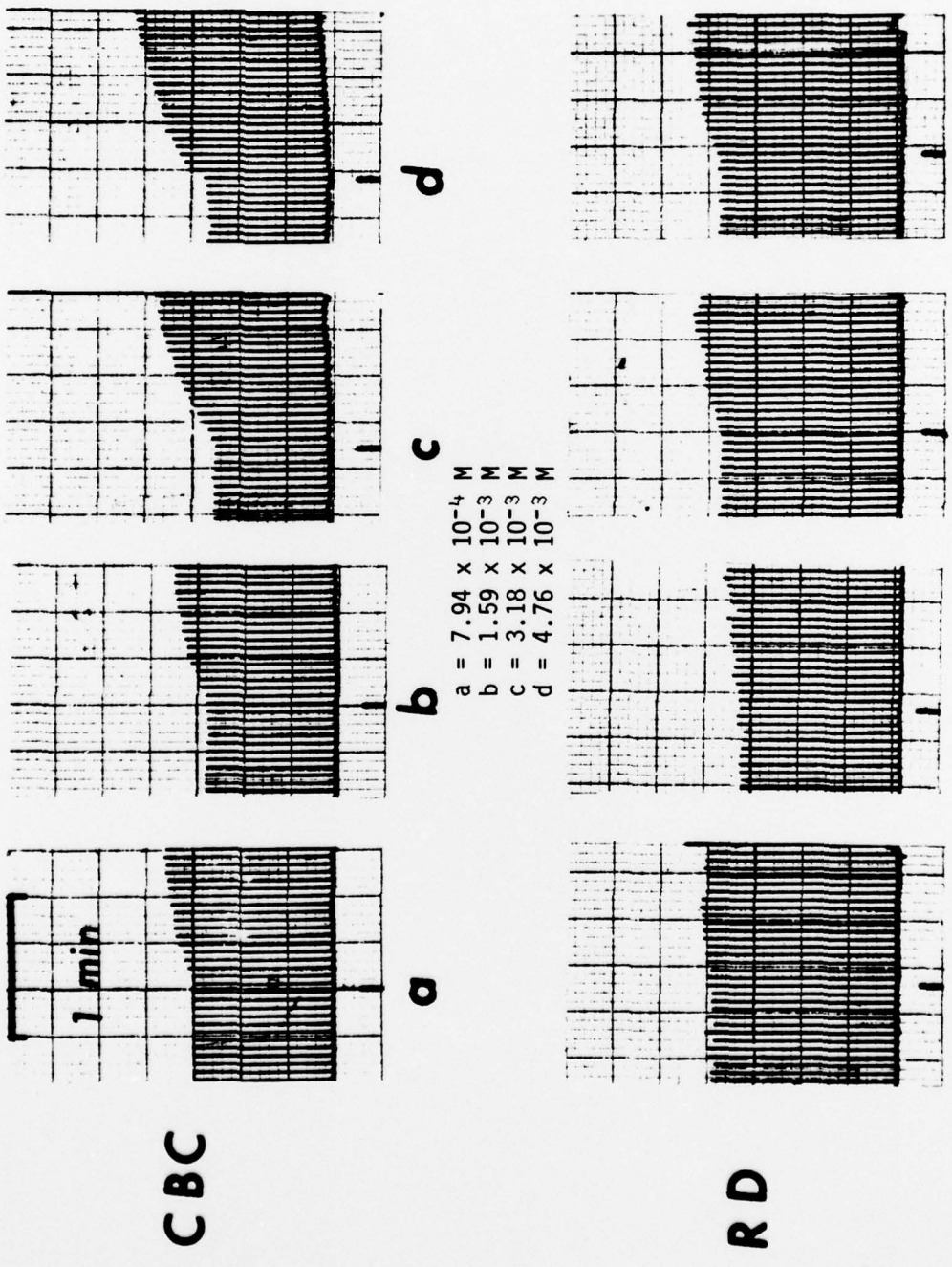


Figure 6: Effect of Various Concentrations of TEA on the Supramaximal Twitch Response in the CBC and RD Preparations.

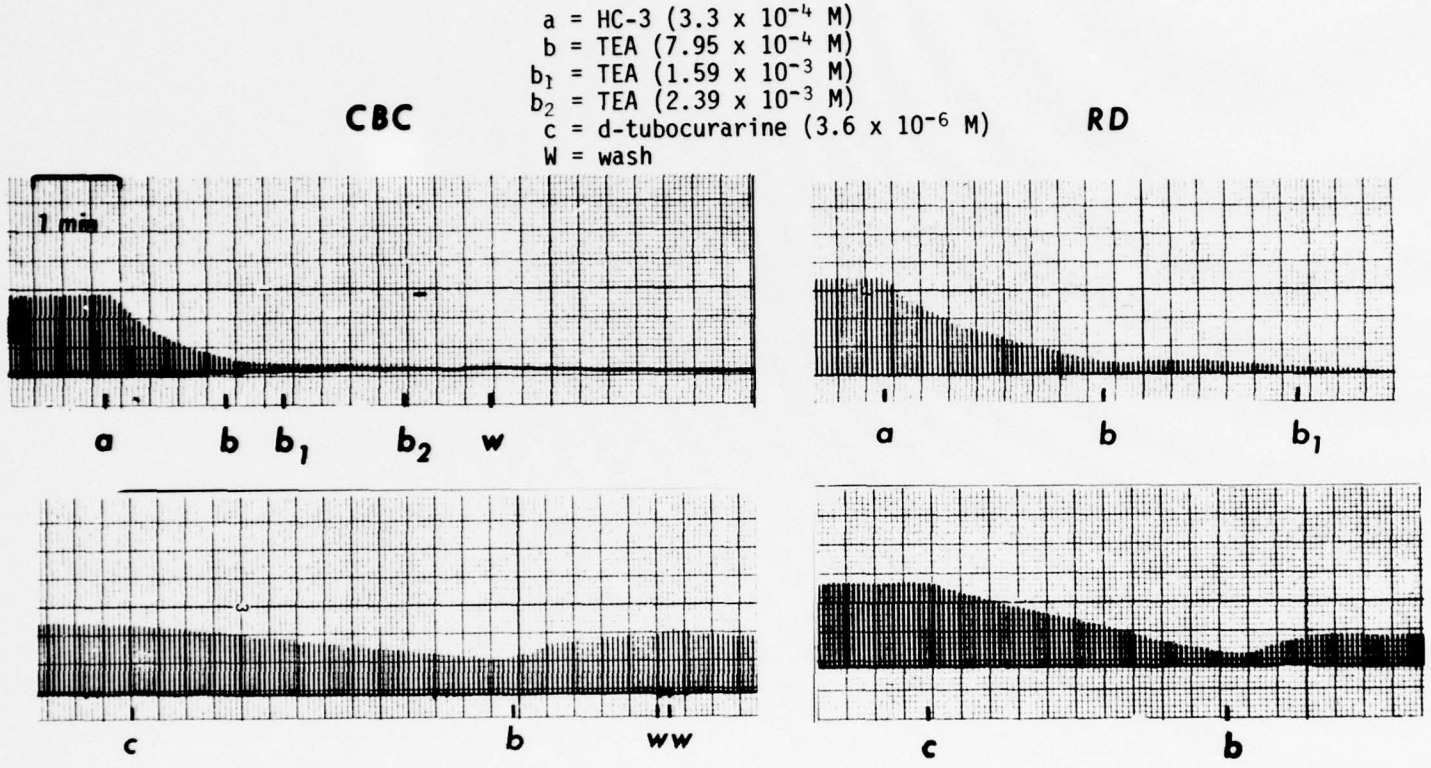


Figure 7: TEA Antagonism of HC-3 and Curare NM Blockade on the CBC and RD Preparations.

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13. ABSTRACT: Enter an abstract giving a brief and factual summary of the document, even though it may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall end with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (TS), (S), (C), (R), or (U).

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